Can the parasitoid *Necremnus tutae* (Hymenoptera: Eulophidae) improve existing biological control of the tomato leafminer *Tuta aboluta* (Lepidoptera: Gelechiidae)?

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Abstract

Necremnus tutae is native to the Mediterranean region where it has been observed in greenhouses parasitizing the invasive *Tuta absoluta* on tomato. The objective of the present study was to determine whether augmentative releases of *N. tutae* can improve existing biological control of *T. absoluta* based on predation by *Nesidicoris tenuis*. Two experiments were carried out, of which the first evaluated different *N. tutae* release rates (1 and 2 *N. tutae* m^{-2} week^{-1}). The parasitoid reduced plant and fruit damage, especially at the higher rate. However, such reduction was considered insufficient given the large numbers of parasitoids needed and still unacceptable level of fruit damage. The second experiment focused on combining the most efficient rate of *N. tutae* of those evaluated during the first experiment, with the pre- and post-planting release of *N. tenuis* and supplemental additions of *Ephestia kuehniella* eggs. Addition of *N. tutae* decreased leaf damage by *T. absoluta* regardless the release method for *N. tenuis*, but the pre-plant release of *N. tenuis* alone was sufficient to prevent fruit damage by *T. absoluta*. This suggested that the addition of *N. tutae* may not be necessary to obtain satisfactory control of *T. absoluta* following pre-plant application of *N. tenuis*, although different options for using *N. tutae* in commercial crops may still be possible.

Keywords: tomato, release rate, release method, predator, *Nesidiocoris tenuis*, tomato pinworm

Introduction

The tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a devastating pest of tomato from South America (Miranda *et al.*, 1998) was first detected in Europe in Spain at the end of 2006. It spread quickly throughout the Mediterranean Basin (Desneux *et al.*, 2010), ravaging tomato crops. Insecticidal control of *T. absoluta* is problematic due, in part, to pesticide resistance (Siqueira *et al.*, 2000; Lietti *et al.*, 2005; Haddi *et al.*, 2012; Campos *et al.*, 2014), its effects on non-target organisms (Biondi *et al.*, 2012*a*, *b*; 2013*a*), market and governmental residue tolerance requirements (COUNCIL OF THE EUROPEAN COMMUNITIES, 2005), and environmental and human health concerns (Pimentel, 2005). Therefore, interest in biological control of this and other pests is increasing.

Different indigenous natural enemies to Europe have been found attacking *T. absoluta* in Europe, which include the eulophid *Necremnus artynes* (Walker) (Hymenoptera: Eulophidae),

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(Ferracini *et al.*, 2012; Urbaneja *et al.*, 2012; Calvo *et al.*, 2013; Zappalá *et al.*, 2013; Abbes *et al.*, 2014; Chailleux *et al.*, 2014*a*; Gebiola *et al.*, 2015). Nevertheless, early reports of *N. artynes* parasitizing *T. absoluta* are now thought, as it is the case of Calvo *et al.* (2013), to refer *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae), which has been found to be the most abundant and widespread species within the *N. artynes* group in Europe (Gebiola *et al.*, 2015). *N. tutae* is an ectoparasitoid, which parasitizes second to fourth instars of *T. absoluta* larvae and inflicts extra mortality by host-killing, i.e. host-feeding and host-stinging (Calvo *et al.*, 2013). Additionally, it exhibits a higher intrinsic rate of increase than *T. absoluta* when reared on tomato (Calvo *et al.*, 2013), altogether indicating potential to control this pest. Integrating the use of such agent alone or combined with other control agents into the present biological control-based integrated pest management programmes (IPM) could provide growers with more options to control *T. absoluta*. However, critical parameters such as rate, timing, and methods or frequency of release under greenhouse conditions needed to implement *N. tutae* for augmentative biological control in protected tomato crops are lacking.

The current standard biological control-based management of tomato pests in the Mediterranean area is based on augmentation of the mirid bug, *Nesidiocoris tenuis* Reuter (Heteroptera: Miridae) (Calvo *et al.*, 2012*a*; Urbaneja *et al.*, 2012; Biondi *et al.*, 2013*b*; Zappalá *et al.*, 2013). This predator commonly appears naturally in tomato and other agricultural crops as well as uncultivated vegetation in the Mediterranean region and the Canary Islands (Malausa & Henao, 1988; Goula & Alomar, 1994; Tavella & Goula, 2001). It is known as an effective natural enemy of whiteflies (Sánchez & Lacasa, 2008; Calvo *et al.*, 2009) and has been used for whitefly control in tomato crops since 2002, and more recently against *T. absoluta* (Calvo *et al.*, 2012*a*; Urbaneja *et al.*, 2012). Two release methods for adult *N. tenuis* are commonly used for augmentative biological control: (1) post-plant application: released 3 or 4 weeks after planting (Calvo & Urbaneja, 2004; Calvo *et al.*, 2009), or (2) pre-plant application: release on tomato seedlings at the plant nursery prior to transplanting (Calvo *et al.*, 2012*b*, *c*). In both cases, *Ephestia kuehniella* eggs Zeller (Lepidoptera: Pyralidae) are provided as a supplemental food during the first weeks after the release (Calvo *et al.*, 2012*a*). Pre-plant application of *N. tenuis* has proven to be very effective for whitefly and *T. absoluta* control, and it is now widely implemented (Calvo *et al.*, 2012*a*). Implementation of *N. tutae* for biological control of *T. absoluta* into the existing programme for commercial tomato greenhouses would be justified if it is proven cost-effective. Nevertheless, multispecies-based programmes can lead to different interactions (Straub *et al.*, 2008), which are expected to benefit biological control if the species belong to different functional groups i.e. species, which do not share a resource/habitat and/or seasonal occurrence (Northfield *et al.*, 2012). *N. tutae* and *N. tenuis* attack different stages of *T. absoluta* (Urbaneja *et al.*, 2009; Calvo *et al.*, 2013), but they could interact negatively through: (1) Kleptoparasitism, the predator feeds on paralyzed host larvae or hostfed larvae by the parasitoid, what induces to ectoparasitoid larval mortality (Chailleux *et al.*, 2014*b*); (2) 'Unidirectional' intraguild predation, the omnivorous predator feeds on parasitoid larvae directly; (3) Competition, either the predator or the parasitoid reduces future prey or host for the other.

For all of this, we conducted the present study whose overall objective was to determine whether the inclusion of *N. tutae*

in the existing IPM programme for tomato would result in better *T. absoluta* control, as well as providing general guidelines for the practical use of *N. tutae* in tomato greenhouses. This was done in two subsequent experiments of which the first reported here (*N. tutae*-alone releases) was aimed at optimizing release rates for *N. tutae* needed to provide good control of *T. absoluta* under a worst case scenario of rapid immigration of the pest into a tomato greenhouse. The second experiment focused on evaluating the potential for improving augmentative biological control of *T. absoluta* by *N. tenuis* with the addition of *N. tutae* (*N. tutae*–*N. tenuis* joint releases) based on the information obtained from the first experiment.

Material and methods

Greenhouse

The experiments were conducted in a multi-tunnel greenhouse located in Vicar (Almeria, Andalusia, Spain), in which 40 walk-in cages were constructed to accommodate the plants and maintain treatments. Twelve and 20 of these cages were used for the first (*N. tutae*-alone releases) and second (*N. tutae*-*N. tenuis* joint releases) experiments, respectively. Walk-in cages were $5 \times 3.5 \times 4$ m^3 (L × W × H) i.e. 17.5 m^2 with walls and ceiling constructed of 'anti-thrips' polyethylene screening with 220×331 μm^2 interstices and supported by heavy wires. Floors were covered with woven 2-mm-thick polyethylene ground cloth and access to each cage was through a zippered doorway. The greenhouse was equipped with a Climatec™ system (Novedades Agrícolas, Murcia, Spain) for temperature and relative humidity (RH) control. Temperature and relative humidity were monitored in four randomly selected walk-in cages with HOBO H8 RH/Temp Loggers (Onset Computer, Bourne, MA, USA). Mean weekly temperature ranged from 19.3 ± 0.96 to 25.6 ± 2.14°C during the first experiment and 23.4 ± 1.54 to 29.1 ± 1.34°C during the second experiment. Mean weekly RH fluctuated from 63.9 ± 2.11 to 72.1 ± 1.97 and 56.4 ± 2.41 to 68.3 ± 2.78% during the two experiments, respectively.

Pests, control agents, and supplemental food

T. absoluta adults used to infest the tomato plants, were collected from a colony maintained on tomato and originally obtained from field collections in several locations within the Province of Murcia (Spain; 37°59′10″N, 1°7′49″W). For each release, pest adults belonged to the same cohort to assure homogeneity in age. *N. tutae* specimens used in the assay were reared in the facilities of Koppert Biological Systems located in Aguilas (Murcia, Spain) on tomato plants using *T. absoluta* as host. Rearing was initiated with more than 200 adults emerging from tomato-leaf samples infested only with *T. absoluta* larvae and collected within the Region de Murcia (Spain), from March to June 2015. For all releases during the experiments, 3-day old or less *N. tutae* adult were used. *N. tenuis* was provided in bottles containing 500 adults (Nesibug™; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands). Eggs of *E. kuehniella* used as supplemental food during the experiment were supplied by Koppert Biological Systems in bottles containing 10 g of eggs (ENTOFOOD™, Koppert Biological Systems, The Netherlands).

Experimental design and procedure

Experiment 1: N. tutae-alone releases

Seeds of tomato, *Solanum lycopersicum* L. (Solanaceae) cv. 'Razymo' (Rijk Zwaan, De Lier, The Netherlands) were planted into 5.4 cm^2 peat moss root cubes. Seedlings with five fully-expanded leaves were transplanted (2 September 2010) into the above-mentioned walk-in cages in 25 l coco peat fiber bags. Twenty-four seedlings were transplanted per walk-in cage, although a typical plant density for tomato cultivation of 2 plants m^{-2} (12 m^2 cage^{-1}) was considered to estimate the number of *N. tutae* adults to be released into cages. Crop cultivation techniques typical for greenhouse tomato cultivation were followed: plants were trained by the main stem to a black polyethylene string tied to a stainless steel overhead wire, secondary shoots were removed and water and fertilizers were supplied as required through a drip irrigation system.

Three treatments were compared in a completely randomized block design with four replicates, in which each of the 12 walk-in cages used constituted a plot, and each block (replicate) consisted in three adjacent cages. Plants from all cages were infested by releasing one *T. absoluta* couple (male × female) per plant every week for 3 weeks since the transplanting date. The treatments were: (1) *T. absoluta*: *T. absoluta* only; (2) 1 *N. tutae* m^{-2}: *T. absoluta* + 1 *N. tutae* m^{-2} (12 adults per walk-in cage released weekly for 7 weeks beginning 2 weeks after planting); and (3) 2 *N. tutae* m^{-2}: *T. absoluta* + 2 *N. tutae* m^{-2} (24 adults per walk-in cage released weekly for 7 weeks beginning 2 weeks after planting). The release schedule for *T. absoluta* was meant to simulate gradual but heavy immigration of the pest into the greenhouse (Calvo *et al.*, 2012*b*). *N. tutae* neither parasitizes nor feeds on first instar *T. absoluta* larvae (Calvo *et al.*, 2013), and thus *N. tutae* releases began 2 weeks after planting coinciding with first availability of second instar larvae of *T. absoluta*. *Tuta absoluta* and parasitoid adults were cooled briefly in a cold room at 8°C for counting before being released into designated walk-in cages at a sex ratio of 1:1.

Experiment 2: N. tutae–N. tenuis joint releases

Five treatments were compared in a completely randomized block design with four replicates. Five adjacent walk-in cages constituted a block (replicate), and again each of the 20 walk-in cages used during the experiment constituted a plot, into which *T. absoluta* was released as above. Treatments were: (1) *T. absoluta*: *T. absoluta* only; (2) *N. tenuis* pre-plant: *T. absoluta* + one *N. tenuis* per two plants released in transplant trays 5 days before planting; (3) *N. tenuis* pre-plant + *N. tutae*: *T. absoluta* + *N. tenuis* released as in treatment 2 + 1 *N. tutae* m^{-2} (12 adults per walk-in cage released weekly for 5 weeks beginning 2 weeks after planting); (4) *N. tenuis* post-plant: *T. absoluta* + one *N. tenuis* per two plants released into walk-in cage the day of planting and (5) *N. tenuis* post-plant + *N. tutae*: *T. absoluta* + *N. tenuis* released as in treatment 4 + 1 *N. tutae* m^{-2} released as in treatment 3. Timing and rate for *N. tenuis* releases were established in accordance with Calvo *et al.* (2012*b, c*) and for *N. tutae* in accordance with results observed during the '*N. tutae*-alone releases' experiment.

For pre-plant inoculation, groups of 24 tomato seedlings at the four-leaf stage grown as above were moved into 'inoculation' cages (1 × 1 × 1.5 m^3). Twelve *N. tenuis* adults were then released into the 'inoculation' cages at a sex ratio of 1:1 after being cooled briefly in a cold room at 8°C for counting. Four paper strips (3 × 1 cm^2) with ca. 0.01 g eggs of *E. kuehniella* glued to one side had been placed inside each inoculating cage to serve as a food source for the mirids. Plants were maintained inside the inoculation cages for 5 days at 25°C, 75% RH and 16:8 (L:D) photoperiod, after which adult *N. tenuis* were removed and the 24 seedlings transplanted into walk-in cages in coco peat fiber bags as above on 2 June 2011. Plants for cages designated for the remaining treatments were maintained under the same conditions during these 5 days, after which they were also transplanted. For post-plant inoculation adult *N. tenuis* were first counted as above before being released into designated walk-in cages at a sex ratio of 1:1. In all cages with *N. tenuis*, eggs of *E. kuehniella* were sprinkled weekly on all plants at a rate of 0.04 g per walk-in cage (Calvo *et al.*, 2012*a, b*), since the transplanting date and for 4 weeks thereafter as a necessary supplement to insufficient *T. absoluta* eggs and larvae for *N. tenuis* nymphs to reach maturity (Urbaneja *et al.*, 2005). Procedures for *T. absoluta* and *N. tutae* release and plant management were the same as those described for the '*N. tutae*-alone releases' experiment.

Sampling

Five randomly selected plants in each walk-in cage were monitored weekly for 11 and 9 weeks after transplanting during the '*N. tutae*-alone releases' and '*N. tutae*-*N. tenuis* joint releases' experiments, respectively, beginning 1 week after the first *T. absoluta* release. *T. absoluta* eggs and larvae were counted on five leaves selected from the upper-mid third of each of the five selected plants (Calvo *et al.*, 2012*b*). Additionally, ten leaves were selected from each of the five selected plants and mined area by *T. absoluta* rated visually as 0, 1, 2, 3, 4, or 5 where 0 was no damage, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–99%, and 5 = 100% of the leaf surface damaged, respectively. Additionally, leaflets were collected weekly from each walk-in cage at all plant strata providing ca. 30 second-fourth instar larvae of *T. absoluta* per walk-in cage. Leaflets were packed in a separate plastic container and labeled by cage. Mines of *T. absoluta* were opened in the laboratory and inspected using a 40× stereoscopic microscope and classified as parasitized (*T. absoluta* larvae with *N. tutae* eggs or larvae), dead (dead larva with no parasitoid eggs, which included host-killing) or alive. Nymphs and adults of *N. tenuis* were counted on three leaves from the upper third of each of the above-mentioned five selected plants (Calvo *et al.*, 2009; Arnó *et al.*, 2010). Leaves were turned carefully to count first *N. tenuis* adults and then nymphs. Finally, fruits from all plants were collected at the end of both experiments, counted, and classified as damaged or not by *T. absoluta*.

Analysis

Incidence of parasitism on *T. absoluta* was expressed as the number of parasitized larvae by *N. tutae* observed per cage divided by the number of live and parasitized larvae per cage. Treatment effects on *T. absoluta* (both experiments) and *N. tenuis* (second experiment) were analyzed using linear mixed effects models ($\alpha = 0.05$), with time (weeks after planting) as random factor nested in blocks to correct for pseudoreplication due to repeated measures (Crawley, 2002).

Table 1. Pair-wise comparison between treatments in the *N. tutae* rate Experiment.

Compared treatments	T. absoluta			N. tutae
	Eggs leaf^{-1}	Larvae leaf^{-1}	Affected area (%)	Total mortality (%)
T. absoluta vs. 1 *N. tutae* m^{-2}	**$F_{1,43} = 20.972$; $P < 0.001$	$F_{1,43} = 0.041$; $P < 0.840$	**$F_{1,43} = 20.826$; $P < 0.001$	$F_{1,39} = 79.627$; $P < 0.001$
T. absoluta vs. 2 *N. tutae* m^{-2}	**$F_{1,43} = 13.576$; $P < 0.001$	**$F_{1,43} = 117.242$; $P < 0.001$	**$F_{1,43} = 30.263$; $P < 0.001$	$F_{1,39} = 81.243$; $P < 0.001$
1 *N. tutae* m^{-2} vs. 2 *N. tutae* m^{-2}	**$F_{1,43} = 124.813$; $P < 0.001$	**$F_{1,43} = 181.126$; $P < 0.001$	**$F_{1,43} = 19.749$; $P < 0.001$	$F_{1,39} = 21.041$; $P < 0.001$

Treatments: (1) *T. absoluta*: *T. absoluta* only; (2) 1 *N. tutae* m^{-2}: *T. absoluta* + 1 *N. tutae* m^{-2} released weekly for 7 weeks beginning 2 weeks after planting; (3) 2 *N. tutae* m^{-2}: *T. absoluta* + *N. tutae* m^{-2} released weekly for 7 weeks beginning 2 weeks after planting. Total mortality included dead larvae by host-killing and parasitism. ** indicates differences between treatments were significant (GLMM, α < 0.05).
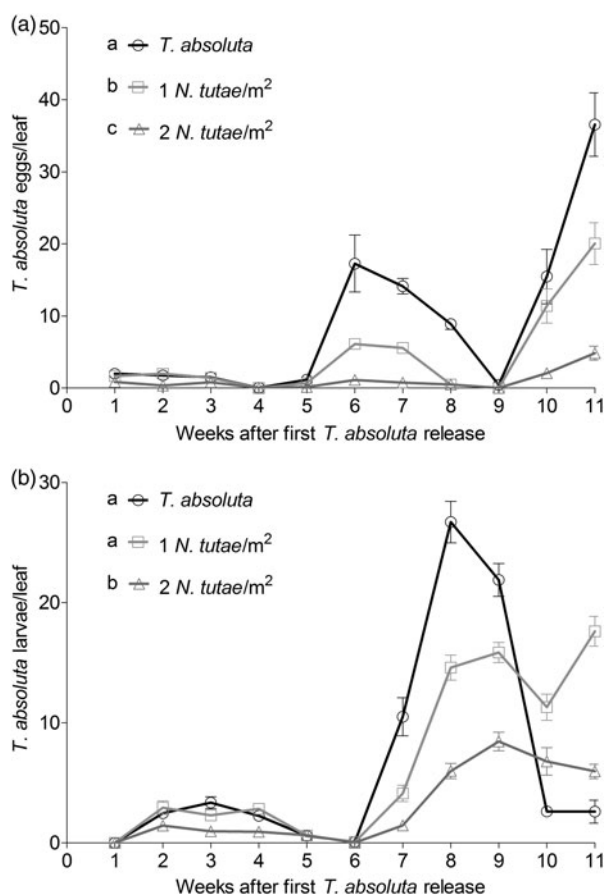

Fig. 1. Mean (±SE) *T. absoluta* eggs (a) and larvae (b) per leaf per week in the '*N. tutae* alone releases' experiment in each treatment: (1) *T. absoluta*; (2) 1 *N. tutae* m^{-2}; (3) 2 *N. tutae* m^{-2}. First *T. absoluta* adults were released the week of planting (week 0) and first *N. tutae* adults 2 weeks later. Evaluations started 1 week after the first *T. absoluta* release. Treatments (legends) with the same letter were not significantly different (GLMM, *P* > 0.05).

Treatments were compared, contingent on a significant model, through model simplification by combining treatments (Crawley, 2002). Differences among treatments in numbers of *N. tenuis* per leaf at each sampling event and proportion of *T. absoluta*-damaged fruits at the end of the experiments were evaluated using a two-way analysis of variance and Tukey's test for mean separation (α = 0.05). Numbers of *N. tenuis* per leaf were log(*x* + 1) transformed, whereas percentages of affected area and damaged fruits by *T. absoluta* were arcsin $\sqrt{(x)}$ transformed prior to analysis to stabilize error variance although untransformed values are given in the text.

Results

Experiment 1: N. tutae-alone releases

Eggs and larvae of T. absoluta

More *T. absoluta* eggs were recorded in cages with the pest only than in cages with parasitoid release (fig. 1a; table 1), with fewest eggs at the higher parasitoid release. Dynamics of *T. absoluta* larvae were similar to those recorded for eggs during almost the entire experiment, though only the higher rate of *N. tutae* significantly reduced larval host (fig. 1b; table 1). At the end of the experiment, plants in the absence of *N. tutae* collapsed due to pest damage and no longer supported larval feeding, whereas plants receiving the lower rate of the parasitoid were still suitable to host *T. absoluta* larvae, thus resulting in a similar abundance of pest larvae compared with the control at the end of the experiment.

Plant and fruit injury by T. absoluta

Leaf area damaged by *T. absoluta* increased continuously in the absence of *N. tutae*, reaching ca. 95% at the end of the experiment (fig. 2a). Parasitoid releases significantly reduced plant damage, especially the higher release rate (table 1). More *T. absoluta*-damaged fruits were found on untreated plants compared with those receiving *N. tutae*, especially at the higher rate ($F_{2,6} = 83.2$; $P < 0.001$; fig. 2b). Nevertheless, nearly 60 and 15% of the fruits were still damaged by *T. absoluta* from plants receiving 1 and 2 *N. tutae* m^{-2}, respectively.

Incidence of parasitism and mortality

Mortality of *T. absoluta* larvae (excluding parasitism) was lowest and close to zero in the absence of *N. tutae*, in contrast to plants receiving the parasitoid, presumably due to host-killing. Consequently, greatest mortality was seen with the higher release rate of *N. tutae* (fig. 3). Incidence of parasitism followed a similar pattern ($F_{1,39} = 14.392$; $P = 0.001$; fig. 3). Thus, the combined effects of host-killing and parasitism resulted in a greater total mortality of *T. absoluta* larvae in cages receiving the higher rate of the parasitoid (table 1).
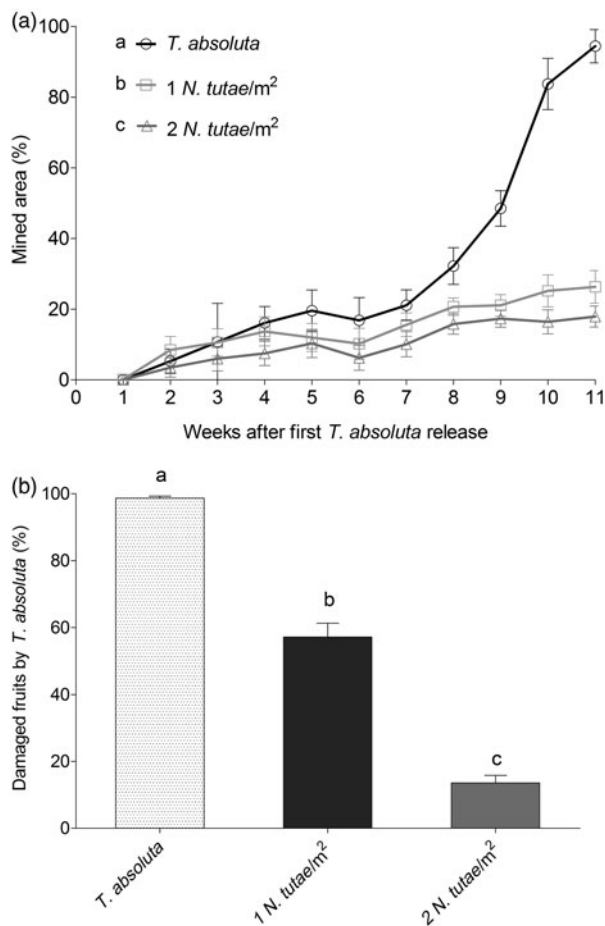
Fig. 2. Percentage (±SE) of mined leaf area by *T. absoluta* per week (a) and mean percentage (±SE) of fruits affected by *T. absoluta* (b) in the 'N. tutae alone releases' experiment in each treatment: (1) *T. absoluta*; (2) 1 *N. tutae* m^{-2}; (3) 2 *N. tutae* m^{-2}. First *T. absoluta* adults were released the week of planting (week 0) and first *N. tutae* adults 2 weeks later. Evaluations of mined leaf area started 1 week after the first *T. absoluta* release and fruit damage was assessed at the end of the experiment when all fruits were collected. Treatments (legends) (a) and columns (b) with the same letter were not significantly different: (a) GLMM, $P > 0.05$; (b) Tukey, $P > 0.05$.

Experiment 2: N. tutae-N. tenuis joint releases

Nesidiocoris tenuis

More *N. tenuis* per leaf were observed on plants receiving the predator pre- vs. post-planting during most weeks of the experiment (Week 1: $F_{3,9} = 14.703$; $P < 0.001$; Week 2: $F_{3,9} = 6.599$; $P = 0.012$; Week 4: $F_{3,9} = 4.241$; $P = 0.006$; Week 5: $F_{3,9} = 4.167$; $P = 0.042$; Week 6: $F_{3,9} = 6.608$; $P = 0.012$; Week 7: $F_{3,9} = 5.271$; $P = 0.023$; fig. 4), and consequently abundance of *N. tenuis* over all 9 weeks was greatest on plants receiving the predator before planting, intermediate when the predator was released alone after planting and lowest in cages receiving the predator after planting with supplementary releases of *N. tutae* (table 2; fig. 4).
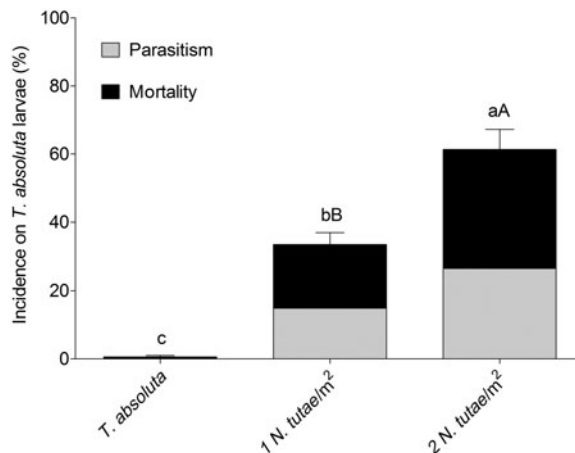

Fig. 3. Overall incidence (±SE) of parasitism and mortality (dead larvae) by *N. tutae* on *T. absoluta* larvae from leaves samples collected in the 'N. tutae alone releases' experiment in each treatment: (1) *T. absoluta*; (2) 1 *N. tutae* m^{-2}; (3) 2 *N. tutae* m^{-2}. Columns with the same small (mortality) or capital (parasitism) letter were not significantly different (Tukey, $P > 0.05$).

Eggs and galleries of T. absoluta

Most eggs of *T. absoluta* were found on plants with *T. absoluta* only (table 2), except for the last week when plants collapsed due to pest attack and no *T. absoluta* eggs were found (fig. 5a). Release of *N. tenuis* reduced *T. absoluta* eggs numbers, especially when released before planting (table 2). Egg suppression was improved under both release scenarios with no difference between them when combined with *N. tutae* (table 2). Pre-plant release of *N. tenuis* plus *N. tutae* was the most effective in reducing *T. absoluta* larvae (fig. 5b; table 2) and the combination of *N. tutae* and post-plant release of the *N. tenuis* was as effective as pre-plant release of the predator alone. Post-plant application of *N. tenuis* alone reduced larval numbers significantly compared with no release, but was the least effective treatment among those receiving beneficial insects.

Plant and fruit injury by T. absoluta

Mined leaf area increased most rapidly on plants receiving only *T. absoluta* (fig. 6a). Release of *N. tenuis* after planting reduced damage, but was less effective than either the combination of post-planting releases of *N. tenuis* plus *N. tutae* or pre-plant releases of *N. tenuis* alone, with similar results from these latter two treatments. The combination of pre-plant releases of *N. tenuis* with supplementary releases of *N. tutae* was most effective in reducing plant feeding by *T. absoluta*. Most fruits were damaged on plants receiving *T. absoluta* only, with intermediate damage in those receiving only the predator after planting and least damage with the remaining treatments, which were not significantly different from each other ($F_{4,11} = 4.997$; $P = 0.014$, fig. 6b).

Incidence of parasitism and mortality

Mortality of *T. absoluta* larvae was mainly observed on plants receiving *N. tutae*, with no significant effects of *N. tenuis* (fig. 7, table 2). These findings reflect low natural mortality of *T. absoluta* larvae and little larval predation by *N tenuis*,
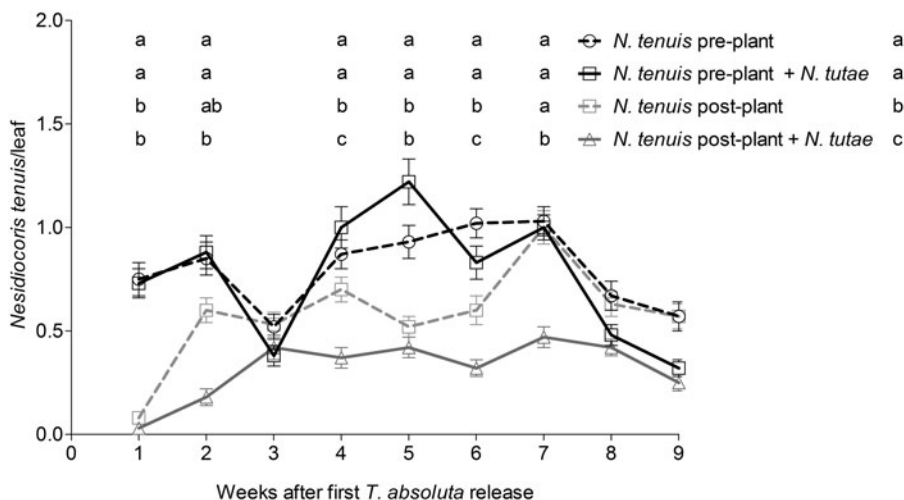
Fig. 4. Mean (±SE) number of *N. tenuis* per leaf per week during the 'Necremnus tutae-N. tenuis joint releases' experiment in each treatment receiving the predator: (1) *T. absoluta*; (2) *N. tenuis* pre-plant; (3) *N. tenuis* pre-plant + *N. tutae*; (4) *N. tenuis* post-plant; (5) *N. tenuis* post-plant + *N. tutae*. *N. tenuis* was released 5 days before or the day of planting in pre-plant or post-plant treatments, respectively. First *T. absoluta* adults were release the day of planting (Week 0) and evaluations started 1 week later. Treatments with the same letter on the right were not significantly different (GLMM, $P > 0.05$), whereas treatments with the same letter on each week were not significantly different in that week (Tukey, $P > 0.05$).

Table 2. Pair-wise comparison between treatments in the *N. tutae* + *N. tenuis* Experiment.

	Statistics				
	Nt	*T. absoluta*			*N. tutae*
Compared treatments	Individuals leaf^{-1}	Eggs leaf^{-1}	Larvae leaf^{-1}	Affected area (%)	Total mortality (%)
Tab vs. Nt post-plant		**$F_{1,35} = 122.33$; $P < 0.001$	**$F_{1,35} = 58.413$; $P < 0.001$	**$F_{1,35} = 63.517$; $P < 0.001$	$F_{1,56} = 0.441$; $P = 0.510$
Tab vs. Nt post-plant + Nect		**$F_{1,35} = 188.01$; $P < 0.001$	**$F_{1,35} = 122.82$; $P < 0.001$	**$F_{1,35} = 72.060$; $P < 0.001$	**$F_{1,56} = 54.327$; $P < 0.001$
Tab vs. Nt pre-plant		**$F_{1,35} = 170.26$; $P < 0.001$	**$F_{1,35} = 120.88$; $P < 0.001$	**$F_{1,35} = 38.021$; $P < 0.001$	$F_{1,56} = 0.027$; $P = 0.871$
Tab vs. Nt pre-plant + Nect		**$F_{1,35} = 187.81$; $P < 0.001$	**$F_{1,35} = 162.74$; $P < 0.001$	**$F_{1,35} = 78.532$; $P < 0.001$	**$F_{1,56} = 50.634$; $P < 0.001$
Nt post-plant vs. Nt post-plant + NAr	**$F_{1,35} = 35.286$; $P < 0.001$	**$F_{1,35} = 186.00$; $P < 0.001$	**$F_{1,35} = 61.000$; $P < 0.001$	**$F_{1,35} = 91.692$; $P < 0.001$	**$F_{1,56} = 48.385$; $P < 0.001$
Nt post-plant vs. Nt pre-plant:	**$F_{1,35} = 15.528$; $P < 0.001$	**$F_{1,35} = 113.99$; $P < 0.001$	**$F_{1,35} = 60.073$; $P < 0.001$	**$F_{1,35} = 92.180$; $P < 0.001$	$F_{1,56} = 0.211$; $P = 0.692$
Nt post-plant vs. Nt pre-plant + Nect	**$F_{1,35} = 9.478$; $P = 0.002$	**$F_{1,35} = 184.54$; $P < 0.001$	**$F_{1,35} = 133.17$; $P < 0.001$	**$F_{1,35} = 107.79$; $P < 0.001$	**$F_{1,56} = 39.980$; $P < 0.001$
Nt post-plant + Nect vs. Nt pre-plant	**$F_{1,35} = 92.356$; $P < 0.001$	**$F_{1,35} = 84.036$; $P < 0.001$	$F_{1,35} = 0.338$; $P = 0.561$	$F_{1,35} = 0.483$; $P = 0.386$	**$F_{1,56} = 55.372$; $P < 0.001$
Nt post-plant + Nect vs. Nt pre-plant + Nect	**$F_{1,35} = 67.807$; $P < 0.001$	$F_{1,35} = 1.340$; $P = 0.247$	**$F_{1,35} = 96.080$; $P < 0.001$	**$F_{1,35} = 168.64$; $P < 0.001$	$F_{1,56} = 1.322$; $P = 0.260$
Nt pre-plant vs. Nt pre-plant + Nect	$F_{1,35} = 0.387$; $P = 0.534$	**$F_{1,35} = 84.036$; $P < 0.001$	**$F_{1,35} = 83.711$; $P < 0.001$	**$F_{1,35} = 206.85$; $P < 0.001$	**$F_{1,56} = 47.291$; $P < 0.001$

Treatments: (1) *Tuta absoluta*: *T. absoluta* only; (2) *N. tenuis* post-plant: *T. absoluta* + one *N. tenuis* per two plants released into walk-in cage the day of planting; (3) *N. tenuis* post-plant + *N. tutae*: *T. absoluta* + *N. tenuis* released as in treatment 2 and 1 *N. tutae* m^{-2} released weekly for 5 weeks beginning 2 weeks after planting; (4) *N. tenuis* pre-plant: *T. absoluta* + one *N. tenuis* per two plants released in transplant trays 5 days before planting; (5) *N. tenuis* pre-plant + *N. tutae*: *T. absoluta* + *N. tenuis* released as in treatment 4 + 1 *N. tutae* m^{-2} released as in treatment 3.** indicates differences between treatments were significant (GLMM, $\alpha < 0.05$). Tab: *T. absoluta*; Nt: *N. tenuis*; Nect: *N. tutae*.

whereas the parasitoid killed *T. absoluta* larvae through both host-feeding and parasitism. Pre-plant release of *N. tenuis* reduced overall incidence of parasitism by *N. tutae* ($F_{1,56} = 5.969$; $P = 0.021$), but larval mortality (excluding parasitism) was no different between the two treatments receiving *N. tutae*: $F_{1,56} = 0.007$; $P = 0.932$).

Discussion

Parasitism and host-killing following releases of *N. tutae* over seven consecutive weeks resulted in decreasing *T. absoluta* larval populations and consequently reduced plant and fruit injury, especially at the higher release rate
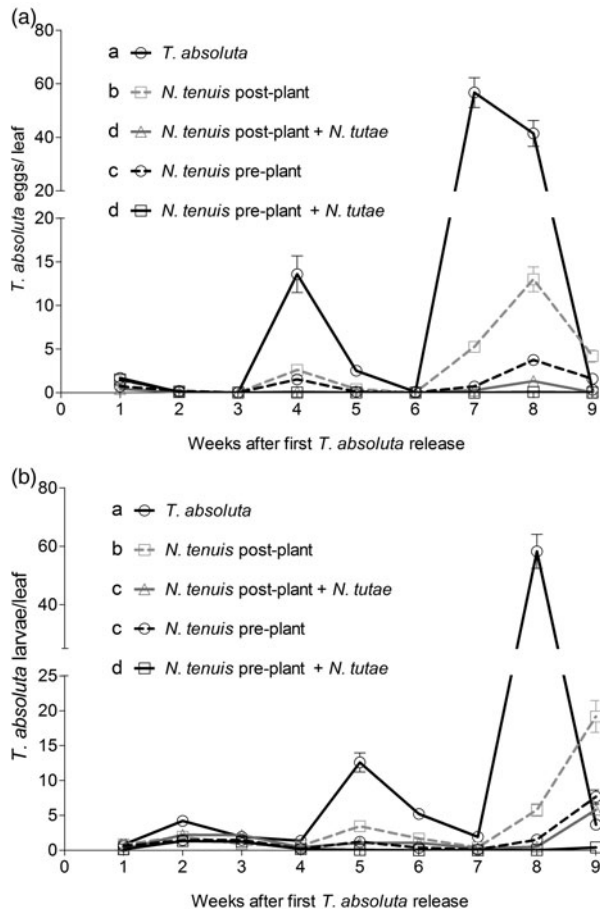
Fig. 5. Mean (±SE) *T. absoluta* eggs (a) and larvae (b) per leaf per week in the '*Necremnus tutae-Nesidiocoris tenuis*' joint releases' experiment in each treatment: (1) *T. absoluta*; (2) *N. tenuis* pre-plant; (3) *N. tenuis* pre-plant + *N. tutae*; (4) *N. tenuis* post-plant; (5) *N. tenuis* post-plant + *N. tutae*. First *T. absoluta* adults were released the week of planting (week 0) and first *N. tutae* adults 2 weeks later. *N. tenuis* was released 5 days before or the day of planting in pre-plant or post-plant treatments, respectively, and evaluations started 1 week after the first *T. absoluta* release. Treatments (legends) with the same letter were not significantly different (GLMM, *P* > 0.05).
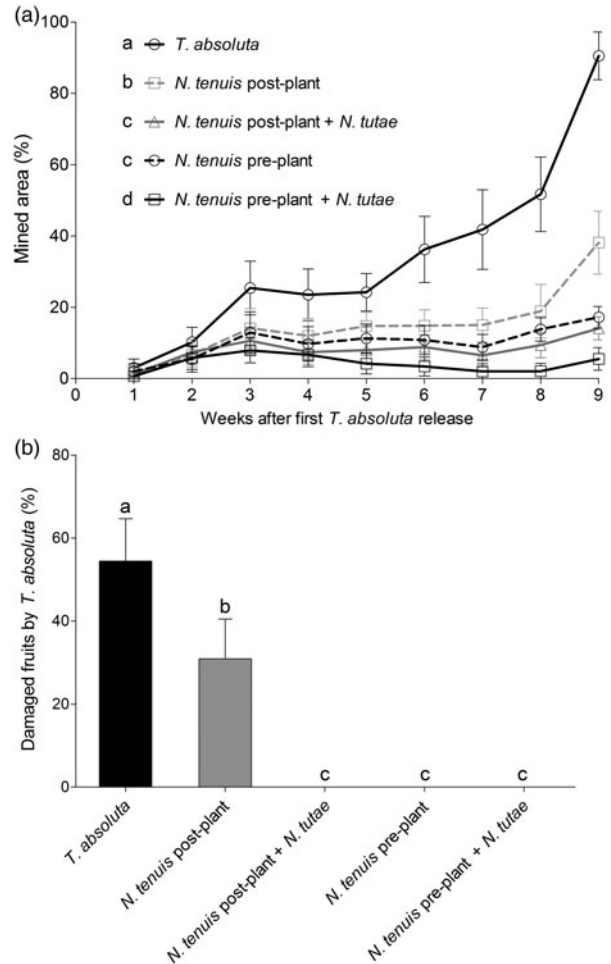

Fig. 6. Percentage (±SE) of mined leaf area by *T. absoluta* per week (a) and mean percentage (±SE) of fruits affected by *T. absoluta* (b) in the '*Necremnus tutae-N. tenuis*' joint releases' experiment in each treatment: (1) *T. absoluta*; (2) *N. tenuis* pre-plant; (3) *N. tenuis* pre-plant + *N. tutae*; (4) *N. tenuis* post-plant; (5) *N. tenuis* post-plant + *N. tutae*. First *T. absoluta* adults were released the week of planting (week 0) and first *N. tutae* adults 2 weeks later. *N. tenuis* was released 5 days before or the day of planting in pre-plant or post-plant treatments, respectively. Evaluations of mined leaf area started 1 week after the first *T. absoluta* release and fruit damage was assessed at the end of the experiment when all the fruits were collected. Treatments (legends) (a) and columns (b) with the same letter were not significantly different: (a) GLMM, *P* > 0.05; (b) Tukey, *P* > 0.05.

(2 wasps m^{-2} week^{-1}). Dead hostfed larvae on plants receiving *N. tutae* exceeded parasitism rates either in the absence or presence of *N. tenuis* (figs 3 and 7 respectively), which correlates with earlier reports (Ferracini *et al.*, 2012; Calvo *et al.*, 2013) that concurred with this finding. While possibly advantageous for pest control, a high host-killing rate is a source of inefficiency for mass rearing. This is particularly important in the current *T. absoluta*-tomato plant based-rearing system for *N. tutae*, which provides the parasitoid with a limited number of suitable hosts for parasitization and which is reduced more by host-feeding. This increases costs per insect and consequently reduces cost-effectiveness. In our study, two *N. tutae* m^{-2} per week during 7 weeks provided more reduction of *T. absoluta* damage, but it was insufficient to reduce plant and fruit damage to an acceptable level (Desneux *et al.*, 2010). Thus, we conclude that *N. tenuis* would probably require

assistance from *N. tenuis* to provide adequate control of *T. absoluta* in commercial tomato greenhouses.

Given that *N. tenuis* can be released either after (post-plant application) or before (pre-plant application) transplanting (Calvo & Urbaneja, 2004; Calvo *et al.*, 2009, 2012a, b, c), both release methods were evaluated in the second experiment, with and without supplementary releases of *N. tutae*. Our results confirmed the superiority of the pre- over the after-plant application of *N. tenuis* both in number of mirids produced during the critical early phase of the crop cycle and ultimately in more efficient reduction of plant and fruit damage by *T. absoluta*. Addition of *N. tutae* to the system increased control of *T. absoluta* with both *N. tenuis* release methods. Results were
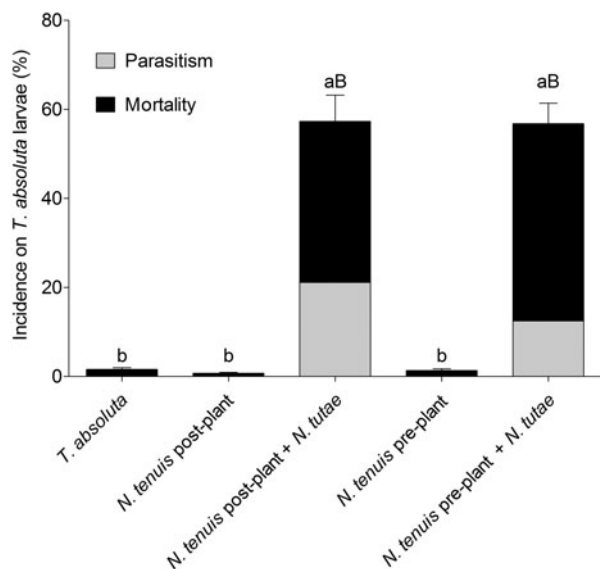
Fig. 7. Overall incidence (±SE) of parasitism and mortality (dead larvae) by *N. tutae* on *T. absoluta* larvae from leaves samples collected in the '*N. tutae* and *Nesidiocoris tenuis*' experiment in each treatment: (1) *T. absoluta*; (2) *N. tenuis* pre-plant; (3) *N. tenuis* pre-plant + *N. tutae*; (4) *N. tenuis* post-plant; (5) *N. tenuis* post-plant + *N. tutae*. Columns with the same small (mortality) or capital (parasitism) letter were not significantly different (Tukey, $P > 0.05$).

comparable in cages receiving the predator after planting and *N. tutae* to cages with *N. tenuis* released before planting only and best when the pre-plant application of the predator was combined with *N. tutae*, which was the only tactic being able to keep defoliation levels under action thresholds (Desneux *et al.*, 2010). Nevertheless, except in cages treated with *N. tenuis* after planting only, excess of action threshold did not result in fruit damage. *N. tutae* exerted an additive effect on control when combined with *N. tenuis* regardless of the release method, killing ca. 60% of second-third instar larvae, whereas almost no larval mortality was seen with the predator alone. *N. tutae* and *N. tenuis* would belong to different functional groups (Northfield *et al.*, 2012), and as expected, their combination provided better control than *N. tenuis* alone released by either method. With both natural enemies present, all immature stages of *T. absoluta* except pupae are subject to attack. Such complementarity could be particularly important at the critical beginning of the crop cycle. Nevertheless, substantial improvement would be needed to justify adding another biological control agent into the system due to pre-plant release of *N. tenuis* alone was sufficient to prevent fruit damage under our experimental conditions, obviating the necessity of *N. tutae*. Differently, Calvo *et al.* (2012*b*) found that the combination of the egg parasitoid *Trichograma achaeae* Nagaraja & Nagarkatti (Hymenoptera: Trichogrammatidae) and/or *Bacillus thuringiensis* Berliner var. kurstaki (Bt) did not increase control of *T. absoluta* over pre-plant release of *N. tenuis* alone. Although Bt has been demonstrated effective against *T. absoluta* either alone or in combination with natural enemies (González-Cabrera *et al.*, 2011; Mollá *et al.*, 2011), it acts on *T. absoluta* larvae primarily when they exit their galleries. Contrarily, *N. tutae* is a specialist parasitoid that can reach *T. absoluta* larvae inside the plant tissue

(and at some extent also outside the plant tissue with high pest densities; FJ Calvo, Personal observation). This provides a better opportunity for *N. tutae* to attack and kill *T. absoluta* compared with Bt, which has little contribution when few *T. absoluta* eggs escape a well-established *N. tenuis* population (Calvo *et al.*, 2012*b*).

Likewise, *T. achaeae*, which naturally occurs in the Mediterranean region (Zappalá *et al.*, 2013), did not improve control of *T. absoluta* when *N. tenuis* was released before planting in nurseries i.e. when the predator was already well established before arrival of *T. absoluta* or *T. achaea* releases began (Calvo *et al.*, 2012*b*). *Macrolophus pygmaeus* (Rambur) (Heteroptera: Miridae), which is biologically comparable with *N. tenuis*, was also found to reduce effectiveness of *T. achaeae* against *T. absoluta*, but not at a greenhouse level (Chailleux *et al.*, 2013, 2014*b*). Contrarily, Desneux *et al.* (2010) found that supplementary releases of *T. achaeae* following release of *N. tenuis* after planting improved control of *T. absoluta*. Nevertheless, attempts to use different *Trichogramma* species against *T. absoluta* in Europe (Zappalá *et al.*, 2013) have provided little contribution to the control of the pest, and thus required periodical inundative releases and/or combination with other control agents, primarily mirid predators (Chailleux *et al.*, 2012, 2014*a*, *b*). Rates, timing, methods, and frequency of natural enemy release as well as synchronization between prey and predator, abiotic factors (humidity, photoperiod, temperature, etc.), and pesticide use can affect control capacity of a natural enemy (Collier & Van Steenwyk, 2004; Stiling & Cornelissen, 2005; Crowder, 2006; Desneux *et al.*, 2007) and thus could help to explain differences among above-mentioned results. Additionally, *N. tenuis* exhibits better life-history traits than *M. pygmaeus* when fed on *T. absoluta* eggs (Mollá *et al.*, 2014), suggesting that *N. tenuis* attacking *T. absoluta* would leave even less room for *T. achaeae* than *M. pygmaeus*.

In a short-term interaction study, Chailleux *et al.*, (2014*a*) observed a strong negative effect of kleptoparasitism on *Stenomesius japonicus* Ashamed (Hymenoptera: Eulophidae), another ectoparasitoid of *T. absoluta* larvae, when combined with *M. pygmaeus*. Similarly, *N. tutae* as *S. japonicus* parasitizes and kills *T. absoluta* larvae by host-killing (Calvo *et al.*, 2013; Chailleux *et al.*, 2014*b*), which reduces future prey for the predator. Nevertheless, *N. tutae* uses oldest *T. absoluta* larval stages for parasitization (Calvo *et al.*, 2013), whereas predation of *N. tenuis* is reduced on these stages (Urbaneja *et al.*, 2009). The predator can also reduce availability of future hosts for the parasitoid (predation) as well as can feed directly on parasitoid larvae (intraguild predation). Consequently, excluding kleptoparasitism of which we have no estimate, a putative reduction of future hosts by unidirectional omnivorous intraguild predation, where *N. tenuis* could feed directly on *N. tutae* ectoparasitoid larvae, or resource competition between both species were probably the most important interactions between the predator and the parasitoid during our study.

In summary, *N. tutae* alone did not reduce plant or fruit damage by *T. absoluta* under an acceptable level, whereas releasing *N. tutae* on plants that had been inoculated with *N. tenuis* provided adequate control. Nevertheless, this combination just slightly improved pest control in leaves over the pre-plant release of *N. tenuis*. Additionally, pre-plant release of *N. tenuis* also provides excellent control of whiteflies (Calvo *et al.*, 2012*c*) and thus, under most situations, this method would be the most cost-effective option for an IPM program in Mediterranean tomato greenhouses, since strategies

involving fewer natural enemy species are often simpler and cheaper. Thus, while our study demonstrates potential for integrating *N. tutae* into biologically-based pest management systems for tomato, the additional cost of such addition has yet to be justified. An efficient rearing method for *N. tutae* might open the door for future inclusion of *N. tutae* within an integrated biological control system. Meanwhile, conservation biological control based on the manipulation of the ubiquitous *N. tutae* could be a feasible sanitary measure against *T. absoluta* in many tomato areas of Europe.

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